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(54) Title: SEPARATION OF HUMAN SERUM ALBUMIN

(57) Abstract

Milk from transgenic bovine animals contains human serum albumin (hSA) and endogenous bovine serum albumin (bSA). The hSA is purified to at least 98 % purity by a separation method which includes affinity chromatography in the presence of a detergent; the affinity ligand being triazine dye molecules. The affinity chromatography step can be incorporated into a protocol to provide essentially pure recombinant hSA as determined by SDS-PAGE and radioimmunoassays.

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SEPARATION OF HUMAN SERUM ALBUMIN

This invention relates to the separation (purification) of human serum albumin (hSA), and can be applied to isolation of hSA from the milk of transgenic animals.

Recent advances in the field of molecular biology allow the production of transgenic animals, i.e. animals containing exogenous DNA in the germ line and somatic cells of an animal introduced by way of human intervention. Differences in the regulation of these foreign genes in different cell types make it possible to promote the differential expression of the foreign gene in a pre-selected tissue, for ease of isolation of a protein encoded by the foreign gene, for desired activity of the gene product in the selected tissues, or for other reasons.

An advantage of transgenic animals and differential gene expression is the production of important proteins in large amounts. Such proteins are typically exogenous to the transgenic animal and may comprise pharmaceuticals, nutrients and the like. However, exogenous proteins are preferably expressed in tissues analogous to those in which they are naturally expressed. For example, exogenous milk proteins are preferably expressed in milk-forming tissues in the transgenic animal. As a result, difficult and wholly new isolation problems are presented because the exogenous protein is present in the same

tissue or bodily fluid as its endogenous counterpart, which often has very similar physicochemical properties.

The use of specific regulatory pieces of DNA makes it possible to induce expression of the foreign DNA in preselected tissues, like the mammary gland. Human proteins may be produced in the milk of other vertebrates, like mice [Gordon et al, Biotechnology 5 (1987) : 1183-1187; Pittius et al, Proc. Natl. Acad. Sci. USA 85 (1988) : 5874-5878; Archibald et al, Proc. Natl. Acad. Sci. USA 87 (1990) : 5178-5182; Simons et al, Nature 328 (1987) : 530-532; Meade et al, BioTechnology 8 (1990) : 443-446], sheep [Simons et al, BioTechnology 6 (1988) : 179-183; Clark et al, BioTechnology 7 (1989) : 487-492], rabbits [Buhler et al, BioTechnology 8 (1990) : 140-143], pigs [Brem et al, Zuchthygiene 20 (1985) : 251-252; Wall et al, Proc. Natl. Acad. Sci. USA 88 (1991) : 1696-1700], and cows [Krimpenfort et al, BioTechnology 9 (1991) : 844-847]. Transgenic cows are especially interesting since they produce, at low cost, large quantities of milk (>10,000 litres/year) containing about 35 gram of protein per litre [Swaisgood, Developments in Dairy Chemistry-1, Ed. Fox, Elsevier Applied Science Publisher, London (1982) : 1-59].

Human serum albumin (hSA) is a protein consisting of 585 amino acids [Minghetti et al, J. Biol. Chem. 261 (1986) : 6747-6757], with a molecular weight of about 68000 daltons. It is the most abundant human plasma protein. One of the main functions of hSA is blood osmotic

regulation. Compared with other blood proteins, hSA is responsible for about 80% of the total blood osmolarity. A second function of hSA is to transport fatty acids, bilirubin, and other proteins present in blood, between adipose tissues and other tissues and organs in vertebrates. hSA can be used to restore blood volume in patients with blood loss or shock.

Large amounts of hSA are required in patient treatment. It is useful to have other sources of hSA besides human blood, since limited amounts of human blood are available. Human blood might also be contaminated with viruses like Hepatitis.

The production of transgenic bovine animals containing a transgene encoding human serum albumin (hSA) targeted for expression in mammary secreting cells is described in PCT Publication No WO 91/08216. However, the purification of human serum albumin is complicated by the presence of endogenous bovine serum albumin (bSA) which is known to be present in bovine milk, and which has physicochemical properties similar to human serum albumin. Since hSA has potential pharmaceutical uses which require highly purified hSA, it is imperative that methods be developed to purify hSA from transgenic milk.

bSA is a protein produced in the liver and leaks from the blood to the milk through the epithelium of the mammary gland. Due to bacterial infections in the udder, cows can suffer from (subclinical) mastitis. This is

correlated with an increased somatic cell count and increased levels of bSA present in milk [Smith et al, J. Dairy Res. 46 (1979) : 547-550; Fox et al, J. Dairy. Sci. 64 (1981) : 2258-2261; Honkanen-Buzalski et al, J. Dairy Res. 48 (1981) : 213-223; Poutrel et al, J. Dairy Sci. 66 (1983) : 535-541; McFadden et al, J. Dairy Sci. 71 (1988) : 826-834].

A number of protocols have been reported for purifying serum albumin from serum.

Thus, GDR Patent Application No DD 213 222 reports the isolation of human serum albumin from human serum using Cibacron Blue F3G-A. Albumin was adsorbed to the dye-cellulose column at pH 5-9, and was desorbed with either 1-2M NaCl or 0.2-1M KSCN. GDR Patent No DD 225 996 reports the isolation of serum albumin from serum by ion exchange chromatography on a Cibacron Blue column. The albumin was eluted with 3.5M NaCl.

In Japanese Patent No JP 63179255, a Cibacron Blue-resin polymer column was reported to bind human serum albumin for radioimmunoassay. The column was contacted with an hSA-containing sample and a reducing agent was added. The column was washed, and contacted with ^{125}I-labelled antibody against hSA. In Japanese Patent No JP 02203933, reporting a method of manufacturing dye-containing affinity adsorbents, bovine serum albumin was bound to a Cibacron Blue-cellulose column.

The scientific literature also reports protocols for the isolation of hSA from plasma using dye conjugates. Travis et al, Biochemical Journal, 157(2) (1976) 301-306, report the use of a Cibacron Blue-Sepharose column to isolate serum albumin from human plasma. The serum albumin is recovered by desorption with 0.2M NaSCN, pH 8.0. Kelleher et al, Journal of Chromatography, 173 (1979) 415-418, report the use of a Cibacron Blue-agarose column to isolate mouse, rat, bovine and human albumin from plasma. Leatherbarrow and Dean, Biochemical Journal, 189 (1980) 27-34, studied the mechanism of binding to Cibacron Blue-Sepharose of albumins from rabbit, horse, sheep, bovine or human plasma. The proteins were eluted from the conjugate with 0.5M NaSCN, pH 8.0.

Metcalf et al, Biochemical Journal, 199 (1981) 465-472, studied the interaction of albumins with immobilized Cibacron Blue. Rat, rabbit, sheep, goat, bovine and human serum albumins were obtained as freeze-dried powders or, in the case of one human sample, isolated from human plasma. Miribel et al, Journal of Biochemical and Biophysical Methods, 16 (1988) 1-16, present a mini-review on the use of dye-ligand affinity chromatography for the purification of human plasma proteins.

Prior to the present invention, purification of hSA involved difficult multi-step procedures. Current isolation methods, while useful to isolate hSA from plasma or serum, are not easily adapted to isolation of

the protein from larger volumes or from other sources, especially from transgenic milk or milk whey where unique separation problems arise in isolating exogenous proteins from, inter alia, similar endogenous proteins.

Purification of hSA from normal human milk (ignoring the complications caused by using transgenic milk as a source) involves the preparation of processed milk or milk whey, by removal of fats and/or casein from whole milk, followed by multi-column chromatographic purification procedures to remove other proteins and separate hSA.

Chromatographic techniques are currently preferred for the purification of serum albumin from milk. This approach reportedly produces a better recovery and higher albumin purity, as well as a lower content of albumin polymers, as compared with ethanol fractionation [Curling (1980), in "Methods of Plasma Protein Fractionation", Curling Ed., Academic Press London, UK; Curling, et al., J. Parenteral Sci. Technol. 36, (1982) 59; Berglof, et al and Nartinache et al, (1982) Joint Meeting IHS-IBST, Budapest]. The specific transport role of hSA as well as its major role in maintaining intravascular osmotic pressure may also be better preserved upon chromatographic purification [Steinbruch (1982), Joint Meeting ISH-ISBT, Budapest].

Protocols for the purification of hSA from blood plasma have been reported before. Especially polysulphonated dyes, like Cibacron Blue, coupled to agarose or sepharose

beads look promising, since they are known to bind serum albumins to a varying extent [Travis et al, Biochem. J. 157 (1986) : 301-306; Kelleher et al, J. Chromat. 173 (1979) : 415-418; Leatherbarrow et al, Biochem. J. 189 (1980) : 27-34; Metcalf et al, Biochem. J. 199 (1981) : 465-472; Gianazza et al, Biochem. J. 201 (1982) : 129-136; Chiggeri et al, Clin. Chim. Acta 145 (1985) : 205-211]. Compared with other albumins, hSA interacts very strong with Cibacron Blue [Kelleher et al, J. Chromat. 173 (1979) : 415-418; Leatherbarrow et al, Biochem. J. 189 (1980) : 27-34; Metcalf et al, Biochem. J. 199 (1981) : 465-472].

This invention is based upon the discovery that by utilizing the greater affinity of hSA for certain triazine dye molecules than serum albumins of other species, together with a novel combination of isolation techniques, it is possible to obtain highly pure human serum albumin. As a result, hSA can now be obtained, for example, from the milk of transgenic animals, and used in pharmaceuticals, nutrition supplements and the like.

The present invention accordingly provides a method of separating human serum albumin (hSA) from a source of said hSA which also includes serum albumin endogenous to a non-human species and at least one other protein normally found in the milk whey of said non-human species, comprising:

(a) contacting said source in the presence of a detergent with a ligand which binds hSA more strongly than said endogenous serum albumin thereby to produce an hSA-ligand complex;

(b) separating said hSA-ligand complex; and

(c) freeing hSA from said separated hSA-ligand complex.

The non-human species can, for example, be bovine.

In another aspect the invention provides the use of a detergent and a ligand exhibiting differential binding characteristics as between hSA and serum albumin from a non-human species, for example, bovine serum albumin (bSA) in the separation of hSA from a source thereof which also includes said non-human serum albumin and at least one other protein normally found in the milk whey of said non-human species.

In accordance with the invention, one possible protocol for purifying hSA from cow's milk is as follows. The milk is first skimmed, followed by acid precipitation to remove caseins. Cation beads (e.g. FAST-S) can, if desired, be added to the whey fraction, to remove lactoferrin, lactoperoxidase, immunoglobulins, and other minor amounts of cationic proteins. The resulting whey is then put on a Cibacron Blue-Sepharose column. Residual binding of bSA can be prevented by, for example, 0.05% Tween-20 and 1% ethanol without a significant loss

of hSA. Complete elution of hSA can be obtained in the presence of 2.5 M KCl, 10% ethanol, 0.05% tween-20, pH 8.0. The protein is essentially pure according to SDS-PAGE and radioimmunoassays.

In the alternative, the whey fraction or other source containing hSA and bSA can be applied directly to the said ligand, and hSA differentially removed therefrom using elution with caprylate or other short chain (e.g. less than 12 carbon atoms) fatty acid, or with a functional equivalent such as, for example, salicylate.

Thus, for example, material containing hSA and bSA may be directly contacted with Blue Sepharose in a buffer to present bSA from binding (e.g. low salt, pH 8.0, about 0.05% Tween-20), and hSA bound to the Blue Sepharose eluted with caprylate. Material remaining bound, e.g. bLF, lactoperoxidase, can thereafter be eluted with a high salt concentration (e.g. 2.5M NaCl or Kcl).

In more general terms, in preferred embodiments of the invention, hSA is purified from the milk of transgenic mammals containing DNA targeting the secretion of hSA into the mammal's milk. Such mammals are preferably bovine. When hSA is intended to be used pharmaceutically, methods resulting in the highest possible purity, i.e. preferably greater than about 98%, and more preferably greater than 99%, are required. The present invention is designed to meet such requirements.

As used herein, "human serum albumin" or "hSA" means a polypeptide having an amino acid sequence substantially as that described by Minghetti et al, Ibid; Lawn et al (1981) Nucl. Acids Res., 9, 6103 and exhibiting an hSA function. Thus, sequence variations are included wherein either one or more amino acid residues have been substituted, inserted or deleted, or the order has been altered, provided said function is retained.

Transgenic milk from an animal engineered to express hSA in its milk is first pre-treated to remove fats to produce skim milk, or fats and casein to produce whey. Bovine milk contains a large amount of fat which is preferably first removed from the milk along with casein to produce a whey fraction. A variety of methods are known to those skilled in the art for removal of fats from milk. Specifically, fats may be removed from milk to produce "skimmed milk" by, for example, filtration or centrifugation or by removing the top layer of fat which develops after cold storage in accordance with traditional dairy practice.

The skimmed or low fat milk is then preferably treated to remove casein. A variety of methods are known to those skilled in the art for removal of casein from the milk. Two of such methods are acid precipitation combined with centrifugation and proteolysis with chymosin. Removal of casein from the milk is preferably be carried out by acid precipitation, to produce whey.

After casein has been removed, a variety of other proteins are still present in the whey or whey fraction. Lactoferrin is one such milk protein, and in some embodiments, the next step for isolation of hSA from whey or a whey fraction is the removal of, inter alia, lactoferrin. However, in accordance with the invention (and as already mentioned), it is also possible to proceed without such isolation if the eventual elution employs caprylate.

Lactoferrin, as well as other cationic whey proteins such as immunoglobulins, lactoperoxidase and lysozyme, may be removed from whey by contacting the whey with a strong cation exchange resin. "Strong cation exchange resins" are defined as those chromatographic resins that have negatively charged groups which are completely ionized over a wide pH range. These groups can bind positively charged molecules (or parts of molecules) which can be exchanged for counterions at increased ionic strength. A variety of such resins are known in the art, and include those having sulphate or sulphite binding groups attached to, for example, Sepharose B. Suitable strong cation exchange resins are MONO S™ or FAST S™ (Pharmacia).

The cation exchange resin may be suspended in the whey or may be used in the form of a column or a bed, or in some other desirable form. The fraction remaining after treatment with the resin is collected as the cationic protein-free whey. The whey may be contacted with the exchange resin and subsequently the cation exchange resin

separated from the cationic protein-free whey, e.g. by centrifugation or gravity filtration.

Having removed the bulk of milk proteins from the whey, the remaining proteins are substantially α-lactalbumin, β-lactoglobulin (A and B), and the serum albumins, hSA and bSA. At this stage, hSA can be separated by contacting with an appropriate ligand, e.g. a dye bound to a support. Useful dye molecules are the triazine dyes, for example Cibacron Blue, Procion Red, Green Fractogel, Ramzol Yellow and Procion Turquoise. Cibacron Blue is preferably used to isolate hSA from endogenous bSA.

As indicated, a triazine dye may be used with the dye bound to a support, i.e. a gel substance or a solid support, for example, Sepharose, paper, silica, or a variety of other substances known to those skilled in the art. Other possible matrices include, for example, dextrans, polyacrylamide, agaarose-polyacrylamide copolymers, cellulose and glass. The support substance or matrix may be formed in a variety of shapes, including columns or beds.

The addition of a detergent (e.g. Tween-20 or caprylate) is important in improving separability of hSA and endogenous bSA. The surfactant is preferably added at a concentration of about 0.05% to 0.2%. The detergent may, for example, be used, at a concentration of about 0.05%.

Tween-20 or other detergent may be added to the whey before contacting with the dye-support complex.

If desired after dilution in buffer (e.g. in Buffer A), contacting the whey with triazine dye bound to a support forms an hSA-dye-support complex. The residual whey can be collected as a flow-through, e.g. at the bottom of a dye-carrying column. The flow-through contains substantially all bSA and substantially no hSA.

The resulting hSA-dye-support complex can then be contacted with a wash solution to remove all loosely bound or non-specifically bound or unbound protein. Following the wash step, hSA may be eluted.

Greater efficiency of elution of bound hSA is often achieved from the dye complexes if ethanol is added to the elution solution (elution peaks are sharper). Thus, the elution solution preferably includes about 5% to 20% ethanol, more preferably about 5% to 15% ethanol. This is, however, not always necessary, for example in the caprylate elution process described elsewhere herein no such addition of ethanol is needed.

The hSA collected in the elution solution may, if desired, be further subjected to desalting and concentration by a variety of well-known methods, such as dialysis, ultrafiltration and freeze-drying. Use of gelfiltration may remove hSA dimers and polymers.

It is therefore possible, by employing the process of the invention, for the first time to obtain purified hSA from the milk of a transgenic animal. Such hSA is useful, for example, in the formulation of pharmaceutical compositions or nutrient supplements. Such compositions and nutrient supplements and their manufacture are other aspects of the present invention. Additionally, the hSA produced by the methods of the invention is useful as a blood expander or plasma expander or the like. Such uses are other aspects of the present invention.

The following description, in the form of Examples, is given further to illustrate the present invention, and is not to be construed as a limitation of the scope of the claims.

In the Examples reference is made to various Figures of the accompanying drawings, and in reading these Examples the following information and basic description regarding these Figures should be taken into account.

Fig. 1 Binding and elution of hSA (A,C) and bSA (B,D) to Blue Sepharose as determined in a radioimmunoassay (see Example 1 below).

Binding experiments (A,B): the Blue Sepharose CL-6B beads (1 g/500 ml) were washed (3x) in 50 mM Tris-maleate buffer having a specific pH and containing different amounts of KCl as indicated. ^{125}I-hSA or ^{125}I-bSA were

added, incubated for 6 hrs, washed with the incubation buffer (5x) and counted.

Elution experiments (C,D): beads were washed (3x) and incubated in the presence of ^{125}I-hSA or ^{125}I-bSA for 6 hrs in 0.1 mKCl, 50 mM Tris-maleate pH 7.0 Beads were washed (4x) with 50 mM Tris-maleate buffers having a different pH and various amounts of KCl, and incubated for 1 hr (rotating) at room temperature. Beads were washed again (5x) with the last incubation buffer and counted. The percentage binding of iodinated serum albumin after elution with various buffers is given on the y-axis.

Tris-maleate buffer can be substituted for Tris-HCl without any change in the binding characteristics of the proteins.

Fig. 2 Elution patters of hSA (A) and bSA (B) on a Blue Sepharose column. Pure protein (100 µg) was loaded on a blue sepharose column pre-equilibrated with 0.1 M KCl, 50 mM Tris-HCl pH 8.0 (buffer A)(1 ml/min). After 10 min, the salt concentration was increased to 0.38 M KCl,, 1.5% ethanol (15% buffer B) for 3 min, followed by an increase to 2.5 M KCl, 10% ethanol, 50 mM tris-HCl pH 8.0 (100% buffer B) for 4 min. Thereafter, the column was re-equilibrated with buffer A.

Fig. 3 Effect of Tween-20 on the elution pattern of hSA (A) and bSA (B) on a blue sepharose column.

Elution conditions are identical as described in the legends of Fig. 2, except 0.05% Tween-20 was added to buffer A, and the flow rate was increased to 1 ml/min. To minimize the hSA-'shoulder' (eluting with 15% buffer B) in Fig 2 A, the first step was decreased to 0.25 M KCl, 1.0% ethanol, 50 mM Tris-HCl pH 8.0 (10% buffer B).

Fig. 4 Elution patterns on a blue sepharose column of (FAST-S treated) cow whey in the absence (A) or presence (B) of hSA.

Raw cow's milk to which pure hSA (1 mg/ml) was added, was defatted, removed of caseins and cationic proteins as described in Example. The resulting whey fraction was diluted (5x) in buffer A (0.1 M KCl, 0.05% Tween-20,50 mM Tris-HCl pH 8.0) and loaded on the blue sepharose 6 Fast Flow column (flow rate 1 ml/min). The first step was in 10% buffer B for 7 min. The final step was in 100% buffer B (2.5 M KCl, 0.05% Tween-20, 10% ethanol, 50 mM Tris-HCl pH 8.0) for 4 min, followed by re-equilibration of the column in buffer A.

Fig. 5 Elution pattersn on a Blue Sepharose column of (non-Fast S treated) cow whey in the absence or presence of hSA (A) or bLF (B).

Raw cow's milk to which either pure hSA (1 mg/ml) or bLF (1 mg/ml) was added, was defatted, removed of caseins, and directly loaded (0.45 ml) on a Blue Sepharose 6 Fast Flow column (flow rate 1 ml/min). The column was washed

with buffer A (0.1 M KCI, 50 mM Tris-HCI pH 8.0, 0.05% Tween-20). After 10 min, the column was washed with 100 mM caprylate, 50 mM Tris-HCI pH 8.0 for 10 min, reequilibrated with buffer A for 6 min, and residual bound proteins were eluted with 2.5 M KCI, 50 mM Tris-HCI pH 8.0, 10% ethanol (10 min).

EXAMPLES

Materials

Blue Sepharose CL-6B, Blue Sepharose 6 Fast Flow, S-Sepharose 6 Fast Flow, CNBr-activated Sepharose, Chelating Sepharose, ConA Sepharose, and Heparin Sepharose were obtained from Pharmacia (Uppsala, Sweden). ssDNA ultrogel was from IBF biotechnics (Villeneuve-la-Garenne, France). Serum albumin from humans (A-3782) and bovines (A-0281), both essentially fatty acid-free and globulin-free, α-lactalbumin, β-lactoglobulin A and B were from Sigma (St Louis, MO). Bovine lactoferrin was purified from bovine milk as described below. Tween-20 (poly-(oxyethylene)20 sorbitan-monolaureate) was from Baker (Deventer, The Netherlands). Chloramine T and $Na_2S_2O_5$ were from Merck (Darmstadt, Germany). The monoclonal antibody against hSA was obtained from Cedar (Hornby, Canada). The polyclonal antibodies against hSA and bSA, the bSA monoclonal antibody (bSA 33), and lectins from Tetraglonolobus purpureas (Tetra), Anguilla anguilla (Ali), and Ulex europaeus (UEA) were from Sigma (St Louis, MO). $Na^{125}I$ (5 Cu/mmol) was obtained from

Amersham (UK). All other reagents were at least analytical grade.

Purification of Bovine Lactoferrin from Bovine Milk

Bovine Lactoferrin was purified from bovine milk by a batch extraction method. Solid NaCl was added to a final concentration of 0.4M and Tween-20 was added to a final concentration of 0.02% (v/v). Sodium phosphate buffer (pH 7.5) was added to 20mM final concentration but final pH was not adjusted to 7.5. Milk fat was removed by centrifugation for 10 minutes at 1600 x g in 500 ml polyallomer tubes in a Beckman JA-10 rotor. Packed S Sepharose™ Fast Flow equilibrated with starting buffer (0.4M Nacl, 20 mM sodium phosphate, pH 7.5, 0.02% Tween-20) was added to the processed milk at a ratio of approximately 1 ml of packed resin beads per 5-10mg of lactoferrin in the processed milk. The mixture was stirred for 20 hours, and the resin beads were isolated by centrifugation at 1600 x g for 5 minutes. Supernatant was removed and the beads were washed three times with one volume of starting buffer. The resin was then poured into a column and washed with one volume of 20 mM sodium phosphate, 0.4 M NaCl, pH 7.5. Lactoferrin is eluted from the column with a gradient of 0.4-1.0M NaCl in 1.25 (times the column) volume of 20mM sodium phosphate, pH 7.5, with a flow rate of 10 ml/min.

The purity of the blF preparation was better than 99% with a recovery rate of 80%. Purity was measured by SDS PAGE analysis and spectroscopy analysis.

Radiolabelling

hSA and bSA were radiolabelled using the chloramine T method, essentially done as described by Hunter and Greenwood [Hunter et al, Nature 194 (1962) : 495-497]. In short: 100 µg of hSA or bSA were dissolved in 150 µl phosphate-buffered saline. Chloramine T (50 µl; 0.4 mg/ml in phosphate-buffered saline and $Na^{125}I$ (10 µl) were added, incubated for 1 min, and the labelling was stopped using $Na_2S_2O_5$ (50 µl; 1 mg/ml in phosphate-buffered saline). As a carrier protein either a 2% bSA solution for hSA labelling or 2% hSA for bSA labelling (100 µl) was added. The sample was loaded on a gel filtration column (25 x 1.5 cm column; 5 ml G25 beads on top of a 35 ml S-300; Pharmacia, Uppsala, Sweden). The column was washed with phosphate-buffered saline, 0.1% Tween-20, 0.3% bSA (or hSA), 1 M NaCl, and 1 ml fraction were collected. The labelling efficiency is >85%.

Anti hSA and bSA PoAb's were first purified before labelling: serum containing the PoAb's were ammoniumsulphate (50%) precipitated, and the supernatants were dialyzed against PBS (overnight). After affinity purification of anti hSA and bSA-coupled Sepharoses respectively, the anti hSA and bSA PoAb's were batchwise incubated with either normal bovine serum-coupled

Sepharose or normal human serum-coupled Sepharose to remove crossreacting Ab's. The PoAb's obtained are called anti hSA PoAb (Ab NBS) or anti bSA PoAb (Ab NHS). Proteins (100 µg) were labelled as described above, except that 2% normal rabbit serum was added as a carrier protein, and the proteins were eluted over a PD10 gelfiltration column in a PBS, 0.3% normal rabbit serum, 0.02% NaN_2 buffer.

Preparation of the various Sepharoses

Ascites containing anti hSA (Cedar) Ab was ammoniumsulphate (50%) precipitated, and the supernatants was dialyzed against PBS (overnight). Anti bSA (BSA33) ascites was loaded with a Protein A Sepharose column in 0.1 M Tris-HCI pH 8.9. The column was first washed with 0.1 M Na-citrate pH 5.5 to remove contaminating Ab's. BSA 33 Ab's were eluted with 0.1 M Na-citrate pH 4.5, and dialyzed against PBS. Serum containing anti hSA or bSA PoAb's were ammoniumsulphate (50%) precipitated, and the supernatants were dialyzed against PBS (overnight). The anti hSA and bSA PoAb's were batchwise incubated with either normal bovine serum-coupled Sepharose or normal human serum-coupled Sepharose to remove crossreacting Ab's.

Antibodies and lectins were coupled to Sepharose beads as follows: CNBr-activated Sepharose (0.5 g) was dissolved in 50 ml 1 mM HCl pH 3.0, and incubated for 15 min (rotating). Beads were washed with the same solution (50

ml), and washed with phosphate-buffered saline (50 ml). Antibodies or lectins were added (~2 mg), incubated overnight (rotating) at 4°C, and washed with phosphate-buffered saline (3 times). Thereafter, the beads were washed with 0.5 M glycine pH 8.5, and incubated in the same solution (50 ml) for 2 hrs. Beads were washed with phosphate-buffered saline (5 times), and finally taken up in 250 ml phosphate-buffered saline, 0.2% Tween-20, 0.02% NaN_3 and stored at 4°C. Metal Sepharoses were prepared as follows: 3 ml of Chelating Sepharose was washed 2 times with (twice distilled) water, 2 times with 1 M NaCl, 50 mM EDTA pH 7.4 and incubated in the same buffer for 15 min (rotating) at room temperature. Beads were washed again with water (6 times). 30 ml of a 30 mM metal chloride solution was added and incubated overnight (rotating) at 4°C. Beads were washed with water (3 times) and washed with phosphate-buffered saline, 0.1% Tween-20, 0.02% NaN_3. The beads were taken up in 200 ml of the last buffer and stored at 4°C.

Albumin Radioimmunoassays

hSA RIA: 0.5 ml Cedar anti hSA monoclonal Ab, coupled to CnBr-activated Sepharose (11 mg protein/g Sepharose/1 L PBS, 0.1% Tween-20), was incubated overnight with 50 µl of a titrated standard hSA solution (5 µg/ml, 2.5 µg/ml, etc) or sample to be tested. After 5 times wash with PBS, 0.02% Tween-20, ^{125}I-anti hSA PoAb (Ab NBS) was added (~20,000 cpm), incubated for 6 hours, again washed (4 times), and radioactivity bound to the beads was

measured. bSA (50 µl, 5mg/ml) does not bind in this assay.

bSA RIA: 0.5 ml anti bSA monoclonal Ab (bSA 33), coupled to CnBr-activated Sepharose (1.3 mg protein/g Sepharose/500 ml PBS, 0.1% Tween-20), was incubated overnight with 50 µl of a titrated standard bSA solution (5 µg/ml, 2.5 µg/ml, etc) or sample to be tested. After 5 times wash with PBS, 0.02% Tween-20, ^{125}I-anti bSA PoAb (Ab NBS) was added (~20,000 cpm), incubated for 6 hours, again washed (4 times), and radioactivity bound to the beads was measured.

Blue Sepharose Radioimmunoassays

Binding experiments: the affinity sepharose beads (0.5 ml) were washed (3 times) with various buffers as indicated in the above description of the Figures. ^{125}I-hSA or ^{125}I-bSA (50 µl; ~10,000 cpm) was added and incubated for 6 hrs (rotating) at room temperature. Beads were washed (5 times) with 1 ml incubation buffer, and radioactivity bound to the beads was measured in a LKB Wallace 1261 multigamma scintillation counter.

Elution experiments: ^{125}I-hSA or ^{125}I-bSA were allowed to bind to the beads for 6 hrs at room temperature (rotating) under optimal conditions (indicated in the above description of the Figures). The beads were then washed (4 times) with 1 ml washing buffer in the presence or absence of various amounts of KCl unless indicated

otherwise, incubated for 1 hr, and washed (5 times) in the last incubation buffer. Radioactivity bound to the beads was determined as described above.

Blue Sepharose 6 Fast Flow Column Chromatography

Proteins were filtered (0.45 µm, Schleicher and Schüll) and applied to Blue Sepharose 6 Fast Flow HR 5/5 column (1 ml of resin) using the FPLC system (Pharmacia). After washing the column with various amounts of buffer A (composition: see Examples), a block or linear gradient of buffer B (composition: see results Examples) was applied to elute bound proteins. The flow rates were 1 ml/min unless indicated otherwise. Peaks were monitored by absorption measurements at 280 nm using a flow cell of 0.5 cm, and computer analyzed using the program FPLC director (version 1.03) of Pharmacia, Uppsala, Sweden.

EXAMPLE 1

Binding of hSA and bSA to Sepharoses

The binding of radiolabelled hSA and bSA to Blue Sepharose and other Sepharoses was determined in a radioimmunoassay (Table 2). Monoclonal and polyclonal antibodies against hSA and bSA coupled to Sepharose beads bind hSA and bSA very specifically. No binding was observed to Glycine- and Heparin Sepharose. The absence of binding to lectin sepharoses was expected, since serum albumins are not glycosylated. Of the metal sepharoses

tested, only Cu^{2+}-Sepharose binds both hSA and bSA. The only Sepharose which has a high affinity for hSA and a lower affinity for bSA, besides the (expensive) antibody Sepharoses, is Blue Sepharose (Table 2 - see below). Thus, Blue Sepharose is an ideal candidate for the separation of hSA and bSA. Blue Sepharose chromatography is also easy to scale up.

The binding characteristics of radiolabelled hSA and bSA to blue Sepharose beads were determined in radioimmunoassays. At a salt concentration of 0.1 M KCl, pH 7.0, about 85% of the ^{125}I-hSA bound to the matrix (Fig. 1A). Under this condition binding of ^{125}I-bSA was about 30% (Fig. 1B). Binding of ^{125}I-hSA could be specifically and completely blocked by "cold" hSA while the inhibitory effect of excess "cold" bSA (100 ug/ml) was only 12% (results not shown).

The interaction of hSA and bSA is partly of an electrostatic nature. Increasing the salt concentration to 2.5 M KCl (at pH 7.0) nearly completely (87%) blocked bSA binding, while reducing hSA binding for 29% (Fig. 1A). However, when hSA is allowed to bind to the beads under optimal conditions (50 mM Tris-HCl pH 7.0, 0.1 M KCl), and the beads are subsequently washed with buffers of increasing salt strength, maximally about 70% of the bound hSA is eluted (Fig. 1C). In a control experiment it was found that NaCl is less efficient in removing the proteins from the beads compared with KCl (Table 3 - see below).

As was also observed by Leatherbarrow and Dean [Biochem. J. 189 (1980) : 27-34], binding of hSA and especially bSA is dependent upon the pH. At low salt concentrations, the effect of the pH (tested from pH 6.0 to pH 8.5) is minimal for binding of hSA (Fig. 1A). At pH 8.5 binding of hSA is somewhat reduced. However, binding of bSA is optimal at pH 6.0 to 6.5 and blocked for 65% at pH 8.5 (Fig. 1A,B).

With an increase in pH the effect of salt on the binding and elution of hSA is clearly reduced, suggesting that hydrophobic sites play an important role in the interaction of hSA with Cibacron Blue (Fig. 1A,C). No such effect is observed with bSA (Fig. 1B,D). To reduce hydrophobic interactions ethanol was added to the buffers. As shown in Table 4, 10% ethanol together with 2.5 M KCl completely eluted hSA and bSA from the beads, while 1% ethanol was less efficient.

These results indicate that at pH 8.0, 0.1 M KCl, hSA maximally binds to Cibacron Blue, while the total binding of bSA is only 15%. In the presence of 2.5 M KCl and 10% ethanol all hSA will elute from the dye. These results were used as a starting point for the separation of hSA and bSA on Cibacron Blue-sepharose columns.

Column Chromatography

As is shown in Fig. 2B, part of the bSA which is injected does not bind to the blue Sepharose 6 Fast Flow column. Unexpectedly, most of the bSA elutes at 1.5% ethanol, 0.38 M KCl (in 50 mM Tris-HCl pH 8.0), while some bSA elutes using 10% ethanol, 2.5 M KCl. This difference compared with the radioimmunoassays is probably due to the different way the assays are performed: a small amount of labelled protein used in radioimmunoassays compared with a relatively large amount of protein in column chromatography; and, in addition, more washing steps are applied in the RIA. hSA strongly binds to the column (Fig. 2A). Most of the hSA is eluted with 10% ethanol, 2.5 M KCl; a minor amount does not bind or elutes at 1.5% ethanol, 0.38 M KCl.

The procedure was optimized the following way: 0.05% Tween-20 was added to the proteins before injection, and to the initial low salt buffer (buffer A) used for equilibration of the column. The non-ionic detergent Tween-20 disrupts hydrophobic interactions. As shown in Fig. 3B, Tween-20 markedly reduces the binding of bSA to the column, since most of the bSA is detected in the flow through. A subsequent 1.0% ethanol, 0.25 M KCl step readily elutes the residual bound bSA. The presence of 0.05% Tween-20 hardly affected the binding of hSA; only a small amount of hSA does not bind or elutes at 1.0% ethanol, 0.25 M KCl (Fig. 3A). This result indicates

that hSA and bSA can be separated using blue Sepharose columns.

Spiking of purified proteins on a Blue Sepharose column

The binding characeteristics of several (purified) proteins on a Blue Sepharose column coupled to a FPLC system (Pharmacia) were investigated in greater detail. 500 µg of hSA and bSA (both essentially fatty acid free/globulin free) and other milk and blood plasma proteins were loaded on the column in buffer A (0.1 M KCI, 50 mM Tris-HCl pH 8.0, 0.20% tween-20) and bound proteins were eluted with a linear gradient (30 ml, 0.2 ml/min) of buffer B (2.5 M KCl, 50 mM Tris-HCl pH 8.0, 10% ethanol). As can be seen in Table 1, human and bovine lactoferrin (hLF and bLF resp.) bind well to Blue: about 0.9 M and 1.4 M KCl is necessary to elute the top fractions from the column. A lactoferrin related molecule, human transferrin, does not bind to the column under these conditions. Bovine lactoperoxidase binds to the column and elutes (topfraction) with about 0.33 M KCl. Binding of hLF, bLF, and bovine lactoperoxidase to Blue, and elution of these proteins with salt, has been reported before (Bezwoda et al, Clin. Chim. Acta 157 (1986) 89-94; Bezwoda et al, Biomed. Chromat. 3 (1989) 121-126; Furmanski et al, J. Exp. Med. 170 (1989) 415-429; Shimazaki et al, J. Dairy Sci 74 (1991) 404-408). For the elution of hSA (topfractions) about 0.43-0.46 M KCl is needed (Table 1).

A human immunoglobulin fraction (IgG) also weakly interacts with Blue and is eluted with low salt concentrations (Table 1). If the column is washed for a prolonged time buffer A, IgG probably will not bind any more. Three major milk proteins, bovine α lactalbumin, β lactoglobulin A and B, do not bind to Blue. The binding of bovine αS casein, β casein, and κ casein is more complex. β casein interacts very weakly with Blue. This interaction can probably be blocked if the column is washed with buffer A for a longer time. Both αS and κ casein proeparations are binding partially to Blue, but interpretation of the elution profiles is difficult since these proteins are contaminated with other milk proteins.

Ethanol in buffer B (2.5 M KCl, 50 mM Tris-HCI pH 8.0 ± 10% ethanol) has small effects on the amount of salt needed for elution of purified proteins: about 6% (hLF), 17% (bLF), and 9% (hSA) more salt is needed to elute these proteins in the absence of ethanol. In general proteins also elute in sharper peaks in the presence of ethanol (~10% sharper; not shown). KCl can be substituted for NaCl, although about 1.4 (hLF, bLF) to 1.6-fold (hSA) more NaCl is needed for elution (not shown).

bSA-Blue interaction was very sensitive for Tween-20 (see also Fig 2 and 3). In the absence of Tween-20, about 40% bSA did not bind to the Blue column. Increasing the concentration of Tween-20 to 0.05% (~ 0.4 mM) increased the percentage of nonbound bSA to about 96%. hSA Blue

interaction was much less sensitive for Tween-20: about 2% hSA did not bind to Blue in the absence of Tween-20, and about 14% did not bind in the presence of 0.05-0.2% Tween-20 in buffer A. If the concentration of Tween-20 in buffer A is raised to 0.5% about 44% hSA does not bind at all, and a further 30% hSA is eluted with only 5% buffer B (0.125 M KCI, 50 mM Tris-HCI pH 8.0, 0.5% ethanol). Tween-20 contains long chain fatty acids groups like laurate, myristate, and palmitate. High affinity binding sites for long chain fatty acids are known to be present on albumin (Ka: 10^6-$10^8 M^{-1}$; review Spector, J. Lipid Res. 16 (1975) 165-179; Richieri et al, Biochem. 32 (1993) 7574-7580; review Carter and Ho, Adv. Prot. Chem. 45 (1994) 153-203).

Interestingly, the short chain fatty acid caprylic acid (octanoate) specifically eluted both hSA and bSA bound to Blue (Table 1; notice that buffer A only contains 50 mM Tris-HCI pH 8.0 to allow binding of bSA to Blue). No effect was found on the other proteins tested (Table 1). The concentrations of caprylate needed to start elution of bSA was 0.3-0.5 mM, while hSA started to elute at 2-8 mM caprylate. Topfractions eluted at 4-5 mM and 14-15 mM caprylate for bSA and hSA respectively. Most likely, caprylate binds very fast to albumin and interferes directly or indirectly with albumin-Blue interaction. Caprylate has been used before in the elution of hSA from Cibacron Blue (Harvey et al, In: Separation of Plasma Proteins. Ed. Curling J.M., Pharmacia Uppsala, Sweden (1983)pp79-88).

Table 1 Binding and elution characteristics of several purified proteins on Blue Sepharose 6 Fast Flow column chromatography.

Protein	% Bound[1]	Elution M KCl[2]	Elution mM caprylate[3]
hSA	>95	0.45	14.5
bSA	0	-	4.5
hLF	100	0.9	-
bLF	100	1.4	-
bLP	100	0.33	-
hTF	0	-	-
IgG	20	~0.17	n.d.
αLac	0	-	n.d.
βLG A	0	-	n.d.
βLG B	0	-	n.d.
αS Cas	27	0.95	n.d.
β Cas	<5?	-	n.d.
κ Cas	72	0.57	-

[1]Binding was determined in buffer A (0.1 M KCl, 50 mM Tris-HCl pH 8.0, 0.20% tween-20). Percentages were determined according to the A280 elution profiles.

[2]Bound proteins were eluted with a linear gradient (30 ml, 0.2 ml/min; or, for caseins, with 1.0 ml/min) of 2.5 M KCl, 50 mM Tris-HCl pH 8.0, 10% ethanol buffer. Values represent the amount of KCl needed for elution. Ethanol has only small effects on the elution profiles (see text).

[3]Proteins were loaded in 50 mM Tris-HCl pH 8.0 (to allow binding of some bSA). The column was eluted with a linear gradient of 0-100 mM caprylate, 50 mM Tris-HCl pH 8.0. - no elution with caprylate; n.d. not determined.

Abbreviations: hLF, human lactoferrin; bLF, bovine lactoferrin; bLP, bovine lactoperoxidase; hTF, human transferrin; IgG, human immunoglobulin; αLac, α lactalbumin; βLG A, β lactoglobulin A; βLG B, β lactoglobulin B; αS Cas, αS casein; β Cas, β casein; κ Cas, κ casein. (hTF, bLP, and all caseins were obtained from Sigma; IgG is affinity purified bovine αhLF IgG from GPE).

Table 2 Binding of hSA and bSA to various affinity Sepharoses.

Affinity Sepharose	Binding (%) hSA	bSA
αhSA MoAb	68	1
αhSA PoAb	93	1
αbSA PoAb	1	91
glycine	0	0
heparin	0	0
Cibacron blue	66	8
Fe^{2+}	1	0
Fe^{3+}	2	2
Cu^{2+}	96	93
Zn^{2+}	1	2
ConA	1	0
ss DNA	1	0
Ali	0	0
UEA	1	0
Tetra	0	0

Binding of ^{125}I-hSA and ^{125}I-bSA (10,000 cpm) to 500 µl of various Sepharoses was determined in either phosphate-buffered saline, 10 mM EDTA, 0.1% Tween-20, 0.05% polybrene for the αhSA MoAb- (Cedar, 0.5 g/500 ml), αhSA PoAb (Ab NBS) (+1% normal bovine serum; 0.5 g/500 ml), and αbSA PoAb (Ab NHS) (+1% normal human serum; 0.5 g/500 ml) Sepharoses. Glycine- (1g/500 ml) Sepharose and Heparin

Sepharose (1 g/500 ml) in phosphate-buffered saline, 10 mM EDTA, 0.1% Tween-20. Blue Sepharose CL6-B (0.5g/500 ml) in 0.1 M KCl, 10 mM Tris-HCl pH 7.0. In 0.02% Tween-20, 10 mM Tris-HCl pH 8.0: Fe^{2+}- (1.7 g/125 ml), Fe^{3+}- (1.7 g/125 ml), Cu^{2+}- (1.7 g/500 ml), Zn^{2+}- (1.25 g/125 ml) Sepharoses. In phosphate-buffered saline, 0.2% Tween-20: ConA- (2 ml beads/250 ml), ss DNA- (2 ml beads/250 ml), Ali- (0.5 g Sepharose/250 ml), UEA- (2 ml beads/250 ml), and Tetra- (2 ml beads/250 ml) Sepharoses. After 6 hrs incubation (rotation) the beads were washed (5x) with incubation buffer and counted. Percentage binding is relative to the total amount of labelled hSA or bSA added.

Table 3. Effect of different salts on the binding and elution of hSA and bSA to Cibacron blue.

	NaCl				KCl			
	Binding (%)		Elution (%)		Binding (%)		Elution (%)	
[salt] (M)	hSA	bSA	hSA	bSA	hSA	bSA	hSA	bSA
0.1	84	29	41*	84	86	35	81	28
0.5	82	18	78	16	82	11	78	9
1.0	79	10	66	8	75	7	57	5
1.5	77	11	56	5	72	6	39	4
2.0	71	8	47	4	65	5	32	3
2.5	69	6	41	2	57	3	19	3

* The sample was partially lost, giving rise to the low value.

Binding experiments: Blue Sepharose CL-6B beads (1 g/500 ml) were washed (3x) with 50 mM Tris-HCl pH 7.0 in the presence of varying amounts of KCl or NaCl as indicated in the figure. ^{125}I-hSA or ^{125}I-bSA was added and incubated for 6 hrs (rotating) at room temperature. Beads were washed (5x) with incubation buffer and radioactivity bound to the beads were measured.

Elution experiments: ^{125}I-hSA or ^{125}I-bSA were allowed to bind to the beads for 6 hrs at room temperature (rotating) under optimal conditions (50 mM Tris-HCl pH 7.0, 0.1 M KCl or NaCl). The beads were then washed (4x) with 50 mM Tris HCl pH 7.0 in the presence of various amounts of KCl or NaCl, incubated for 1 hr and washed (5x) with incubation buffer. Radioactivity bound to the beads was measured.

Table 4 Effect of ethanol and salt on the binding and elution of hSA and bSA to Cibacron Blue.

	Binding (%)				Elution (%)			
	1% EtOH		10% EtOH		1% EtOH		10% EtOH	
[KCl] (M)	hSA	bSA	hSA	bSA	hSA	bSA	hSA	bSA
0.1	64	12	61	10	54	9	48	6
0.5	53	5	49	4	40	3	26	1
1.0	46	3	42	3	28	2	10	1
1.5	41	2	37	2	20	2	6	1
2.0	39	2	30	2	15	1	5	1
2.5	36	2	21	1	12	1	2	1

Binding experiments: Blue Sepharose CL-6B beads (1 g/500 ml) were washed (3x) with 0.1 M NaPi pH 7.0 in the presence of varying amounts of KCl and ethanol. ^{125}I-hSA or ^{125}I-bSA was added and incubated for 6 hrs (rotating) at room temperature. Beads were washed (5x) with incubation buffer and radioactivity bound to the beads was measured.

Elution experiments: ^{125}I-hSA or ^{125}I-bSA were allowed to bind to the beads for 6 hrs at room temperature (rotating) under optimal conditions (0.1 M NaPi pH 7.0, 0.1 M KCl). The beads were then washed (4x) with 0.1 M

NaPi pH 7.0 in the presence of various amounts of KCl and ethanol, incubated for 30 min and washed (5x) with incubation buffer. Radioactivity bound to the beads was measured.

Control experiments using a 0.1 M KCl, 0.1 M NaPi pH 7.0 buffer in the absence of ethanol, indicated that 79% hSA and 46% bSA bound to the beads, and after elution with the same buffer 79% hSA and 40% bSA remained bound to the beads.

EXAMPLE 2

This Example describes a way to purify hSA spiked in a raw cow's milk, but more efficient ways (omitting S-Sepharose) are given in subsequent Examples.

Purification of a hSA fraction from hSA-spiked raw bovine milk

Freshly received raw bovine milk was aliquoted and frozen at -80°C. After thawing, either 1 mg/ml hSA alone, 1 mg/ml bSA + 1 mg/ml bovine lactoferrin, a combination of hSA, bSA, and bovine lactoferrin, or only phosphate-buffered saline was added to 10 ml milk, vortexed, and incubated for 30 min at room temperature. Milk was defatted by centrifugation (15,000 rpm, 15 min) at 10°C. The skimmed milk was brought to pH 4.7 with HCl, incubated for 30 min at 40°C, and centrifuged (15,000 rpm, 30 min) at 4°C. The pH of the supernatants was adjusted

to 6.0 with solid Na_2HPO_4 (final concentration 50 mM), and again centrifuged (15,000 rpm, 10 min) at 4°C. To the supernatant was added 2 ml of twice in water diluted S-Sepharose Fast Flow beads (FAST-S), incubated (rotation) for 30 min at room temperature, and centrifuged (1000 rpm, 2 min). The resulting whey fraction was diluted 5 times in buffer A (see description of the Figures, above), filtered (0.45 µm, Schleicher and Schüll), and 2 ml was injected on a Blue Sepharose 6 Fast Flow column as described above.

EXAMPLE 3

Purification of hSA from cow's milk

hSA (1 mg/ml) was added to raw cow's milk in the presence or absence of excess bSA and bovine lactoferrin (both 1mg/ml). After 30 min incubation at room temperature the milk was defatted. The skimmed milk was brought to pH 4.7 to precipitate the caseins. The resulting whey fraction was brought to pH 6.0, and a strong cation exchange resin (FAST-S) was added batchwise to remove cationic proteins like lactoferrin, lactoperoxidase, immunoglobulins, and other cationic proteins present in minor amounts. Both human and bovine lactoferrin are known to bind strongly to Cibacron Blue [Bezwoda et al, Clin. Chim. Acta 157 (1986) : 89-94; Bezwoda et al, Biomed. Chromat. 3 (1989) : 121-126; Furmanski et al, J. Exp. Med. 170 (1989) : 415-429; Shimazaki et al, J. Dairy Sci. 74 (1991) : 404-408].

In the remaining whey fraction α-lactalbumin and β-lactoglobulin (A and B) are still present. However, pure α-lactalbumin and β-globulin A and B do not bind to Blue Sepharose 6 Fast Flow under the conditions used. Thus, the remaining whey fraction was diluted with 4 volumes of buffer A (0.1 M KCl, 0.05% Tween-20, 50 mM Tris-HCl pH 8.0) and directly put on the Blue Sepharose column. A representative run of control whey (in the absence of hSA) is shown in Fig. 4A. Most proteins do not bind to the column; a minor amount elutes at 1% ethanol, 0.05% Tween-20, 0.25 M KCl pH 8.0. This peak partly contains some bSA (results not shown). What the other protein(s) are is unknown at present. However, some whey proteins elute from the column using 2.5 M KCl, 10% ethanol, 0.05% Tween-20. To prevent this, the procedure can be optimized by increasing the length of 0.25 M KCl, 1.0% ethanol step, and/or variations in the (amounts of) detergents and/or salt in buffer A.

In whey containing hSA, one peak elutes at 10% ethanol, 2.5 M KCl (Fig. 4B). This peak will be contaminated with other proteins like bSA. To obtain complete separation, the procedure can be optimized as mentioned above, or samples can be rerun on Blue Sepharose.

EXAMPLE 4

More methods for the purification of hSA from cow's milk

hSA (1 mg/ml) was added to raw cow's milk and incubated overnight at 4°C. As a control, raw milk was spiked with either 1 mg/ml bSA or bLF, or not spiked at all. The milk was defatted by low speed centrifugation (3000 rpm, 10 min), and caseins were removed by high speed centrifugation (17,000 rpm, 60 min). Samples were filtered (0.45 µm) and loaded on a Blue Sepharose column coupled to a FPLC system (Pharmacia). Buffer A contained 0.1 M KCl, 50 mM Tris-HCI pH 8.0, 0.05% Tween-20 to prevent binding of (most) bSA to the column. Bound proteins were first eluted with 100 mM caprylate, 50 mM Tris-HCl pH 8.0 (blockwise). Proteins which did not elute with caprylate were eluted with a buffer containing 2.5 M KCI, 50 mM Tris-HCl pH 8.0, 10% ethanol (blockwise).

As shown in Fig 5A, control (unspiked) whey shows an elution pattern in which most proteins do not bind to the column, a small amount of protein elutes with caprylate, probably representing residual bound bSA, and a significant amount of protein elutes with the 2.5 M salt step. Proteins eluting with high salt probably are bLF, lactoperoxidase, and immunoglobulins (and maybe some residual caseins). The same elution profile was obtained for hSA-spiked milk, except that caprylate now elutes the

bound hSA (Fig 5A). Calculations of the amount of hSA in this A280 peak (corrected for the A280 extinction coefficient (1cm 0.1%) of hSA of 0.53) indicated that >90% of the hSA was bound to the column and could be eluted with caprylate. If the small amount of protein eluted with caprylate with control whey is (residual) bSA, it can be calculated (A 280 peak corrected for the A280 extenction coefficient (1cm 0.1%) of bSA of 0.667) that about 20 µg bSA is present in this fraction. Although accurate determinations still have to be made (RIA's), these experiments indicate that hSA purified from cow's milk will contain less than 5% bSA.

SDS-PAGE analysis (12.5%, Fastgel) of hSA-spiked milk eluted with caprylate from a Blue column showed the predominant hSA monomer and some dimers (<5%). The monomer-dimer ratio was equal to the (Sigma) purified hSA preparation used for the spiking experiments, indicating that the Blue column binds both the monomers and dimers. The caprylate-eluted hSA still contains minor contaminations of bSA (control whey) and some lower MW proteins.

The elution pattern of bSA-spiked whey compared with control whey shows a small increase in the amount of protein eluted with caprylate (not shown). If this increase is solely due to spiked bSA, it can be calculated that this amount of bSA is <10% of the amount loaded on the column (quantitative RIA's still have to be done). bSA binding can probably be dimished by a more

extensive wash with buffer A, or small variations in the amount of Tween-20 and/or salt, or by the addition of mM amounts of caprylate.

The elution pattern of bLF-spiked whey is exactly the same as unspiked control milk, except that now bLF elutes with the high salt step (Fig 5B). According to the elution profiles about 54% bLF could be recovered in this step (A280 peak corrected for the A280 extenction coefficient (1cm, 0.1%) of apo-bLF of 1.1). The remaining bLF is probably removed in the casein precipitation steps, since bLF will likely bind to κ caseins.

Although RIA's (for hSA and bSA) still have to be done, it seems that whey containing hSA can be directly contacted with Blue Sepharose beads under appropriate conditions to separate hSA from bSA and other proteins in a single chromatography step. Contaminations can probably be removed by rerunning on a Blue column followed by gelfiltration.

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The results from the above Examples indicate that hSA binds much more strongly to Cibacron Blue than bSA.

Binding of bSA was more susceptible to variations in pH, salt strength, Tween-20, and caprylate compared with hSA. Several sites have been located on hSA involved in the

binding of metals, fatty acids (long chain), bilirubin, and several other organic molecules (reviewed by Carter and Ho, Adv. Prot. Chem. 45 (1994) 153-203): domain IIA is the primary site for the binding of bilirubin; domain IIIA (together with IIA) the primary site for the binding of small heterocyclic and aromatic carobxylates; and the high affinity binding sites for long chain fatty acids are thought to be located in domains IB and IIIB, and maybe IIIA, although controversy remais about the exact location of these last 3 sites. It has been suggested that bilirubin interfers with hSA-Blue interation while having no effect on bSA-Blue interaction (leatherbarrow and Dean, Biochem. J. 189 (1980) 27-34). However, the same group could not reproduce these results (Metcalf et al, Biochem. J. 199 (1981) 465-472). Apparently this site is not involved in the interaction of serum albumin with the Blue dye. It has been reported that the binding of bSA to Blue could be prevented to a large extent by preincubation of albumin with the long chain fatty acids palmitate, myristate, and laurate, while the binding of hSA was much less affected (Leatherbarrow and Dean, Biochem. J. 189 (1980) 27-34; Metcalf et al, Biochem. J. 199 (1981) 465-472). Our results of hSA purified from hSA-spiked cow's milk (~15 µM) on Blue sepharose confirm these findings. Since the free fatty acid concentration in cow's whey, primarily long chain fatty acids, is about 30-125 µM (reviewed by Edelsten, In: Meat Science, Milk Science, and Technology. Eds. H.R. Cross & A.J. Overby. Elsevier, Amsterdam (1988) pp. 175-199), and baring in mind that the fatty acid-albumin interaction occurs in

milliseconds (as measured by the quenching of tryptophane fluorescence; Richieri et al, Biochem. 32 (1993) 7574-7580), it can be estimated that theoretically all or most high affinity fatty acid binding sites on the spiked defatted hSA molecule are occupied before contact with the Blue dye. Still, nearly all hSA (>90%) binds to the Blue column (see Examples).

Compared with hSA, binding of bSA to Blue was also much more sensitive to the detergent Tween-20 (less than ~400 µM Tween-20 already nearly completely blocks binding of bSA to Blue; our results). Tween-20 probably binds to albumin through its fatty acid side chain (primarily laurate and myristate). These results suggest that bSA binds to Blue via one of the high affinity long chain fatty acid binding sites. Binding of hSA does not occur primarily via these sites, but hSA-Blue interaction might be affected by conformational changes in hSA by binding of high concentrations of long chain fatty acids and Tween-20.

Interestingly, bSA-Blue interaction was completely blocked with 4-5 mM caprylate, and hSA-Blue interaction was completely blocked with 14-15 mM caprylate (see Examples). This effect is specific for hoese serum albumins, since binding of the other (milk) proteins tested (bLF, hLF, bovine lactoperoxidase, and transferrin) to Blue is caprylate-insensitive. Elution of hSA from Blue Sepharose by the short chain fatty acid caprylate has been reported before (Harvey et al, In:

Separation of Plasma Proteins. ed. Curling J.M. Pharmacia Uppsala, Sweden (1983) pp. 79-88; own results), and the Blue dye reduces the binding of ^{125}I-caprylate to hSA (Lagercrantz and Larson, Biochem. J. 213 (1983) 387-390). These results suggest that domain IIIA is directly or indirectly involved in the binding of both albumins to Blue. This site is also thought to be the primary site for the binding of a variety of organic molecules like salicylate and tryptophan (Carter and Ho, Adv. Prot. Chem. 45 (1994) 153-203).

Complete elution of hSA from Cibacron Blue was accomplished with NaSCN [Travis et al, Biochem. J. 157 (1986) : 301-306; Kelleher et al, J. Chromat. 173 (1979) : 415-418; Leatherbarrow et al, Biochem. J. 189 (1980) : 27-34; Metcalf et al, Biochem. J. 199 (1981) : 465-472]. However, prolonged treatment of hSA with NaSCN was reported to induce dimers and oligimers, which are thought to bind less well to Cibacron Blue (Leatherbarrow et al, Biochem. J. 189 (1980) :27-34]. Travis et al found that 2.4% of the albumin recovered were dimers; commercial albumin (Sigma) even contained more dimers plus other minor contaminants [Travis et al, Biochem. J. 157 (1986) : 301-306]. Commercial albumin is obtained by Cohn's fractionation involving ethanol precipitation steps [Cohn et al, J. Amer. Chem. Soc. 68 (1946) : 459-475]. In the procedure we use to bind and elute hSA spiked to cow's milk, the amount of dimers and oligomers as determined by SDS-PAGE is the same as non-spiked hSA.

Thus, the procedure does not induce dimer/oligomer formation of hSA.

A lot is known about the composition of milk from humans and various animals such as cows, pigs and goats. A main milk protein fraction is the family of caseins, with variations in amounts and composition among the different species: ~80% of the total amount of proteins in cow's milk are caseins, while this figure in human milk is ~25% (for reviews, Hambraeus, In: Nutrition Abstracts and Reviews in Clinical Nutrition-Series A, Wiley Ltd (1984) pp 219-236; Lonnerdal, B. Amer. J. Clin. Nutr. 42 (1985) 1299-1317). Caseins can be easily removed from the milk, by either simple sedimentation, isoelectric precipitation at pH 4.6, or treatment with chymosin, which, by limited proteolysis, induces coagulation of casein micelles [Swaisgood, Developments in Dairy Chemistry-1, Elsevier Applied Science Publisher, London (1982) : 1-59]. The resulting whey fraction consists primarily of α-lactalbumin, β-lactoglobulin A and B, serum albumin, lactoferrin, immunoglobulins, and lactoperoxidase. Ionic interaction chromatography is an efficient way to specifically extract anionic or cationic proteins from solutions. It is known that cation exchangers like carboxymethyl-cellulose, MONO-S or FAST-S beads specifically and efficiently bind lactoferrin, lactoperoxidase, and immunoglobulins [Bezwoda et al, Clin. Chim. Acta 157 (1986) : 89-94; Bezwoda et al, Biomed. Chromat. 3 (1989) : 121-126; Furmanski et al, J. Exp. Med. 170 (1989) : 415-429; Ekstrand et al, J.

Chromat. 358 (1986) : 429-433]. The remaining proteins in the whey can be separated on alkyl chain reverse phase columns [Pearce, Aust. J. Dairy Technol. 112? (1983) : 114-117], or on anion columns [Humphrey et al, New Zealand J. Dairy Sci. Tech. 19 (1984) : 197-204; Andrews et al, J. Chromat. 348 (1985) : 177-185]. β-lactoglobulin A and B strongly bind to the anion exchanger MONO-Q, as do α-lactalbumin and serum albumin [Humphrey et al, New Zealand J. Dairy Sci. Tech. 19 (1984) : 197-204; Andrews et al, J. Chromat. 348 (1985) : 177-185]. Given the differences in elution profiles, it is technically possible to separate serum albumin from the two other proteins. However, salt gradients are necessary to purify albumin, with no separation of the human and bovine species. The anion exchange step is not strictly necessary in the purification of human serum albumin from (transgenic) bovine milk, since neither lactalbumin nor lactoglobulin bind to Cibacron Blue. To remove salts and organic solvents, Cibacron Blue purified hSA can be subjected to dialysis, ultrafiltration, or gel filtration. If detergents like Tween-20 are not removed using these procedures, the method of Chen [J. Biol. Chem. 242 (1967) : 173-181], used for defatting serum albumins, might be applicable. The whole purification procedure is relatively easy to scale up.

Alternatively, preincubation of the whey fraction with Fast S Sepharose can be omitted. The bovine whey containing (transgenic) hSA can be directly incubated (batchwise) with Blue Sepharose in a buffer containing

about 0.1 M salt, pH 8.0, 0.05% Tween-20 to prevent bSA binding. Bound hSA can be eluted with caprylate (preferably 1-100 mM), and bound bovine lactoferrin, lactoperoxidase, residual immunoglobulins can be eluted with 2.5 M KCI (or NaCl), 50 mM Tris-HCl pH 8.0 (see also examples below). Strictly speaking it is not necessary to remove caprylate bound to albumin since this compound is often added to albumin for stabilization during heat treatment (removal of virusses in the albumin preparation). If it is necessary to remove contaminating bSA, caprylate probably has to be removed (e.g. the procedure of Chen, J. Biol. Chem. 242 (1967) 173-181) from the albumin sample first before being rerunned on Blue Sepharose.

The normal concentration of bSA in bovine milk is around 0.1-0.2 gr/L [Smith et al, J. Dairy Res. 46 (1979) : 547.550; Fox et al, J. Dairy Sci. 64 (1981) : 2258-2261; Honkanen-Buzalski et al, J. Dairy Res. 48 (1981) : 213-223; Poutrel et al, J. Dairy Sci. 66 (1983) : 535-541]. Given an annual demand for hSA of over 100,000 kg/year for parenteral use in the USA, it can be calculated that about 10,000 milk-producing hSA-transgenic cows, with an expression of about 1 g hSA/L, are necessary to meet the US market. [Expression levels of hSA in milk of transgenic mice of 2.5 gr/L [Shani et al, Transgenic Res. 1 (1992) : 195-208] and more [Hurwitz et al, Int. Patent No WO 93/03164 (1993)] have been reported.] Thus, to produce hSA in an economically feasible way, the concentration of hSA in the milk will be much higher than

the concentration of bSA, which facilitates hSA purification.

CLAIMS:

1. A method of separating human serum albumin (hSA) from a source of said hSA which also includes serum albumin endogenous to a non-human species and at least one other protein normally found in the milk whey of said non-human species, comprising:

(a) contacting said source in the presence of a detergent with a ligand which binds hSA more strongly than said endogenous serum albumin thereby to produce an hSA-ligand complex;

(b) separating said hSA-ligand complex; and

(c) freeing hSA from said separated hSA-ligand complex.

2. A method of claim 1, wherein said endogenous serum albumin is bovine serum albumin (bSA).

3. A method of claim 1 or claim 2, wherein said source is transgenic milk whey or a transgenic milk whey fraction.

4. A method of claim 3, wherein the transgenic milk whey or milk whey fraction is subjected to a cation exchange resin to remove lactoferrin, lactoperoxidase, immunoglobulins, and other cationic proteins.

5. A method of any one of claims 1 to 4, wherein said ligand is a triazine dye.

6. A method of claim 5, wherein said triazine dye is Cibracon Blue.

7. A method of any one of claims 1 to 6, wherein said detergent is Tween, optionally Tween-20.

8. A method of any one of claims 1 to 7, wherein the separation of hSA-ligand complex in step (b) is achieved by using said ligand in immobilised form.

9. A method of claim 8, wherein said immobilized form comprises a chromatographic material having said ligand bound thereto.

10. A method of claim 9, wherein said chromatographic material is Sepharose.

11. A method of claim 9 or claim 10, wherein the freeing of step (c) is effected by contacting said hSA-ligand complex with an elution solution.

12. A method of claim 11, wherein the elution solution includes ethanol.

13. A method of claim 12, wherein the elution solution contains about 5% to 15% ethanol.

14. A method of any one of claims 1 to 13, wherein said at least one other protein is selected from lactoferrin, lactalbumin and lactoglobulin.

15. A method of claim 14, wherein said source is treated prior to step (a) substantially to remove said at least one other protein.

16. The use of a detergent and a ligand exhibiting differential binding characteristics as between hSA and serum albumin from a non-human species, for example, bovine serum albumin (bSA), in the separation of hSA from a source thereof which also includes said non-human serum albumin and at least one other protein normally found in the milk whey of said non-human species.

17. The use of claim 16, wherein said source is transgenic milk whey or a transgenic milk whey fraction.

18. The use of claim 16 or claim 17, wherein said ligand is a triazine dye.

19. The use of claim 18, wherein said triazine dye is Cibacron Blue.

20. The use of any one of claims 17 to 19, wherein the detergent is a short chain fatty acid, optionally having less than 12 carbon atoms, or a functional equivalent thereof.

21. The use of claim 20, wherein the fatty acid is caprylate.

22. The use of claim 20, wherein the functional equivalent is salicylate.

23. A process for separating hSA from a material which also includes serum albumin from a non-human species, optionally bSA, comprising contacting said material with a ligand which exhibits differential binding characteristics as between hSA and said serum albumin from a non-human species thereby to form a complex, and freeing hSA from said complex by treatment with a short chain fatty acid or equivalent thereof.

24. A process of claim 23, wherein said short chain fatty acid has less than 12 carbon atoms.

25. A process of claim 24, wherein said short chain fatty acid is caprylate.

26. A process of claim 23, wherein said equivalent thereof is salicylate.

27. A process of any one of claims 23 to 26, wherein said ligand is a triazine dye.

28. A process of claim 27, wherein said triazine dye is Cibracon Blue.

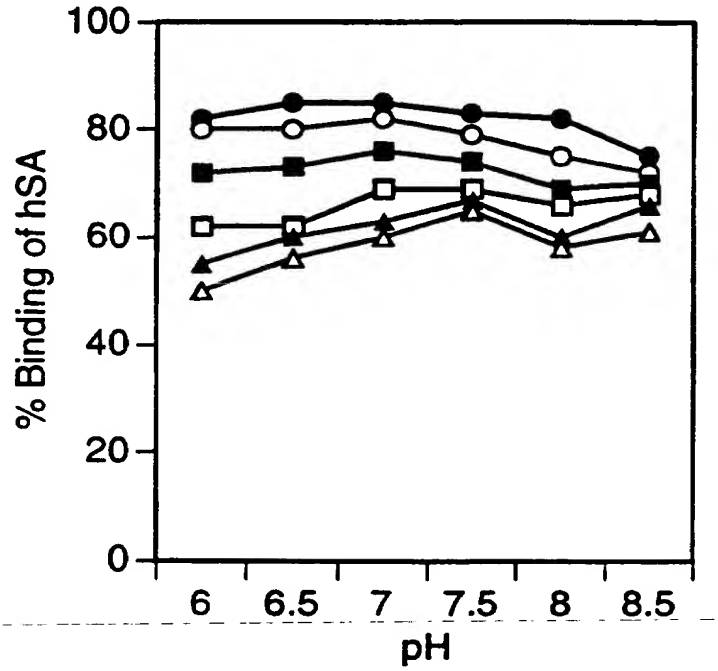
29. A process of any one of claims 23 to 28, wherein said ligand is in immobilized form.

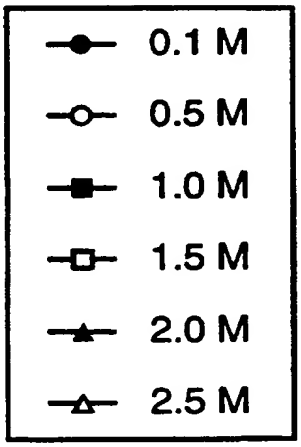
30. A process of claim 29, wherein said immoblized form comprises a chromatographic material having said ligand bound thereto.

31. A process of claim 30, wherein said chromatographic material is Sepharose.

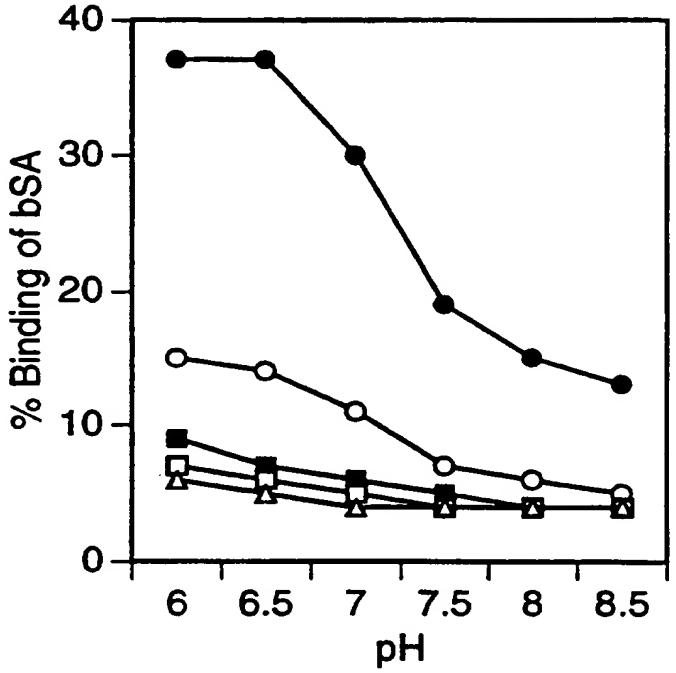
32. A process of claim 30 or claim 31, wherein said short chain fatty acid or equivalent thereof is used to elute hSA from said chromatographic material after formation of said complex.

33. A process of claim 32, wherein after elution of hSA said serum albumin from a non-human species is eluted using high salt conditions.





Figur 1A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30
20
10
0
% Binding of bSA
6
6.5
7
7.5
8
8.5
pH

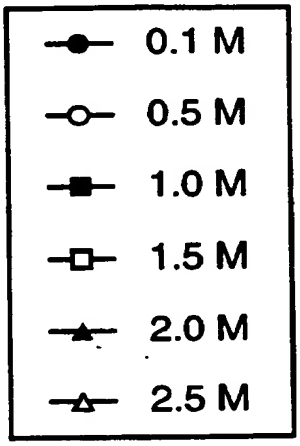

0.1 M
0.5 M
1.0 M
1.5 M
2.0 M
2.5 M

Figure 1B

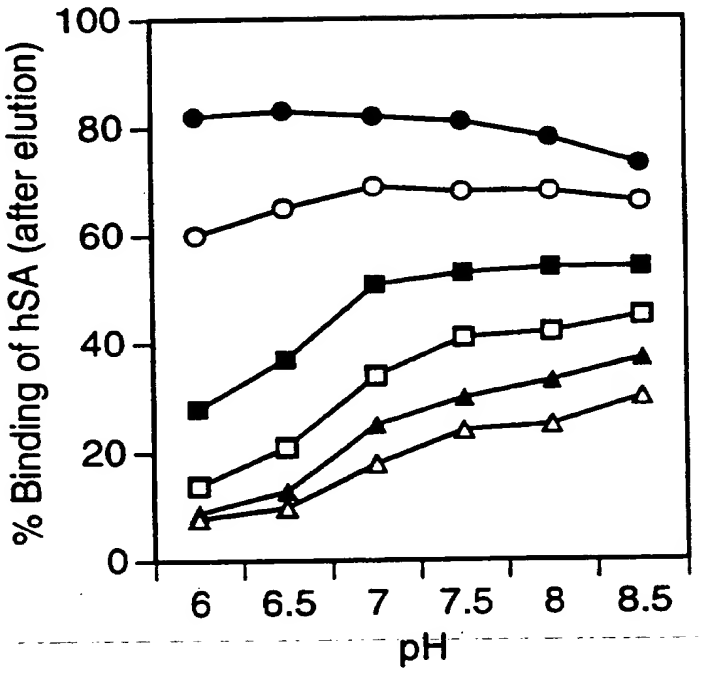


Figure 1C

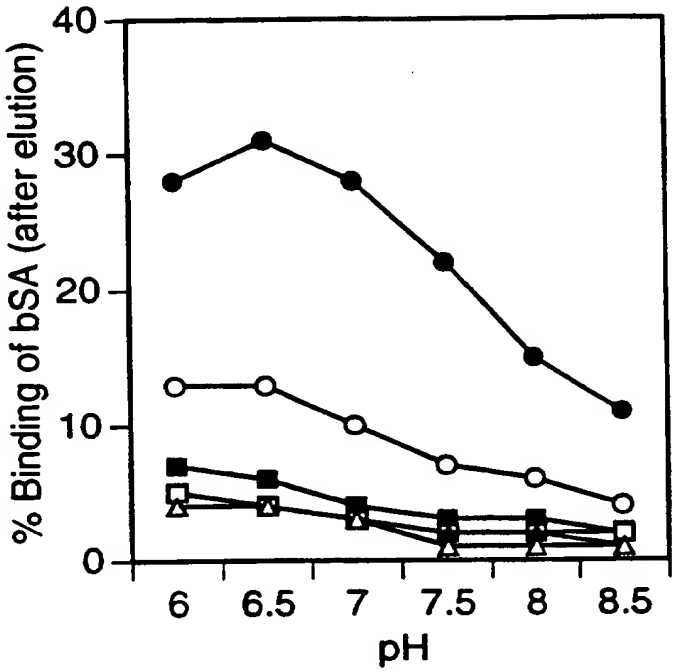
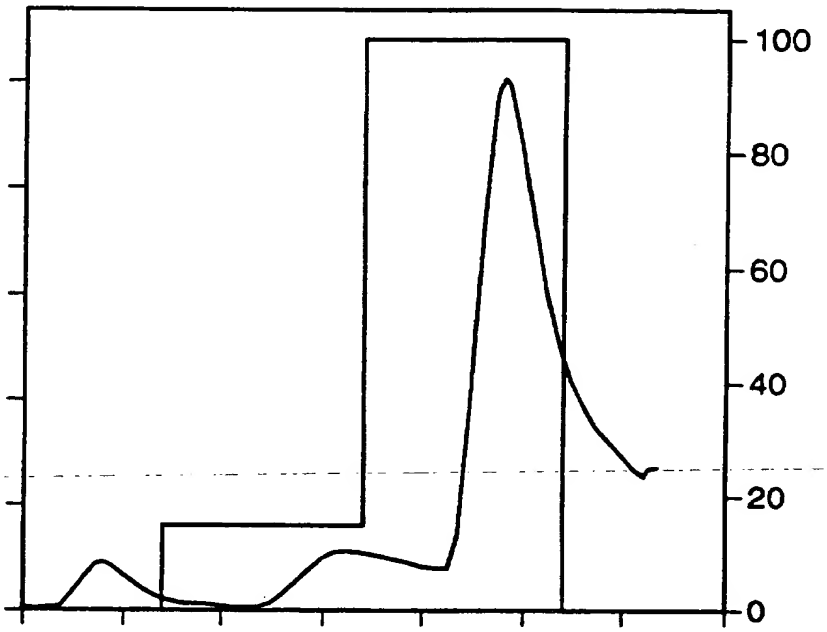
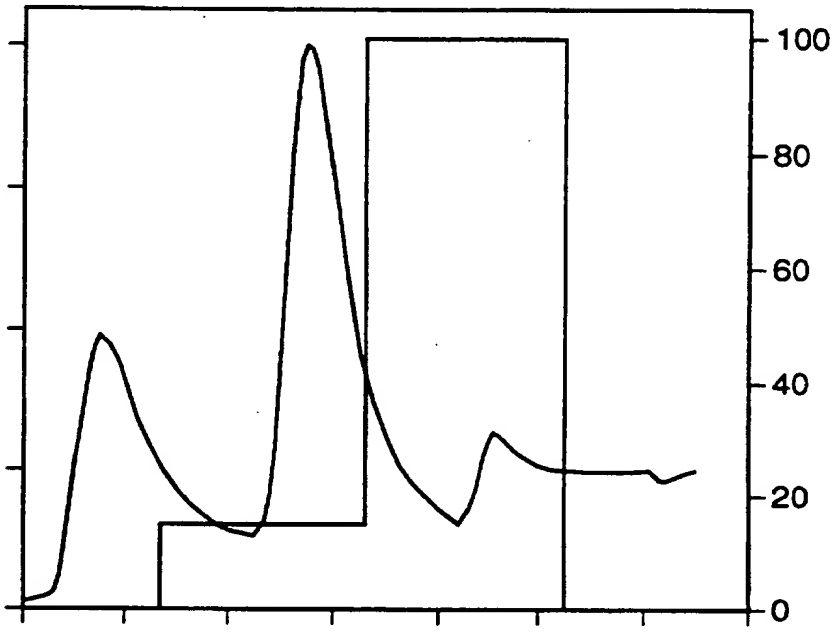


Figure 1D



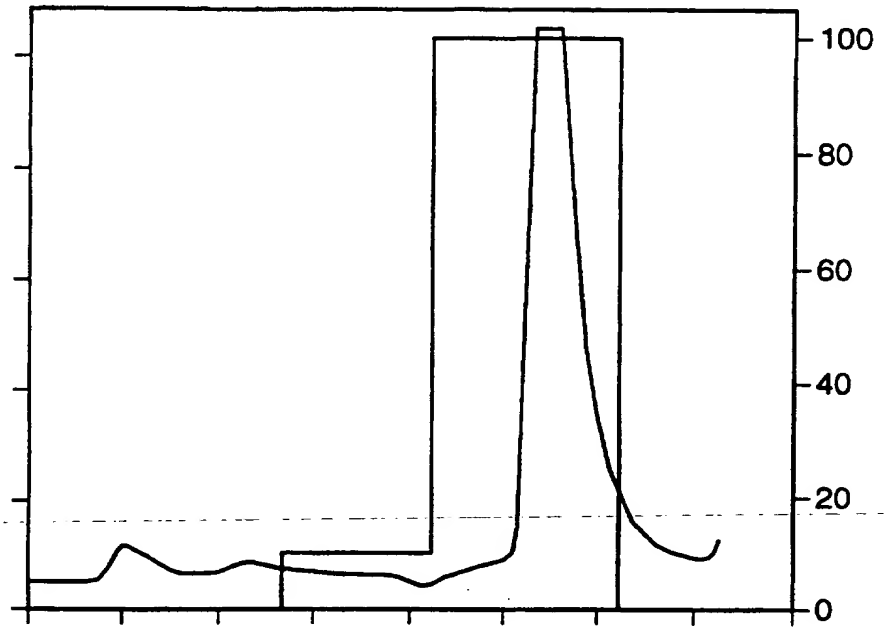
hSA

Figure 2A



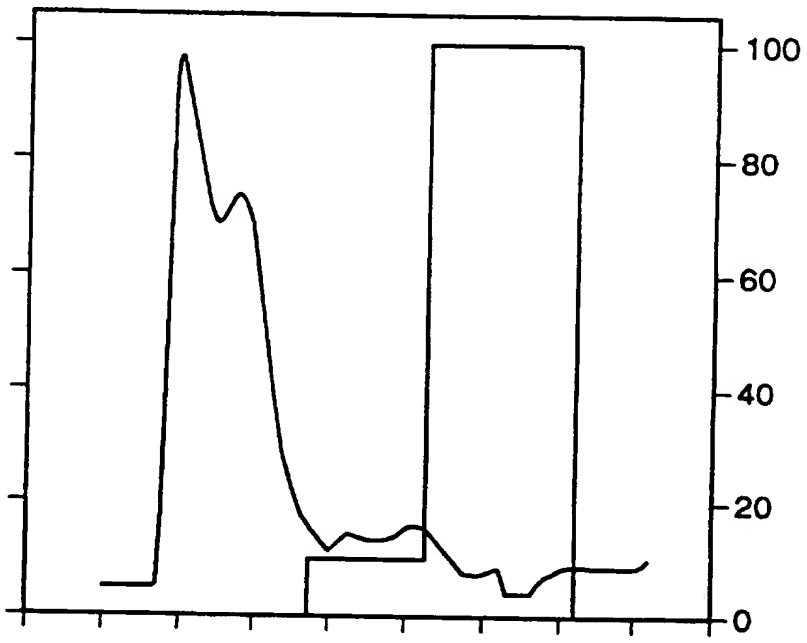
bSA

Figure 2B



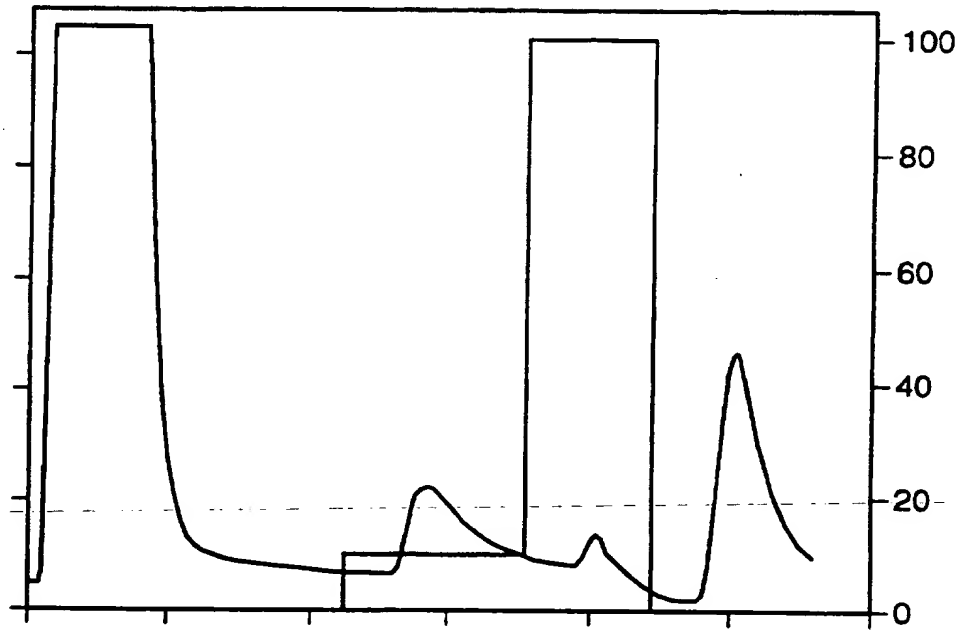
hSA

Figure 3A



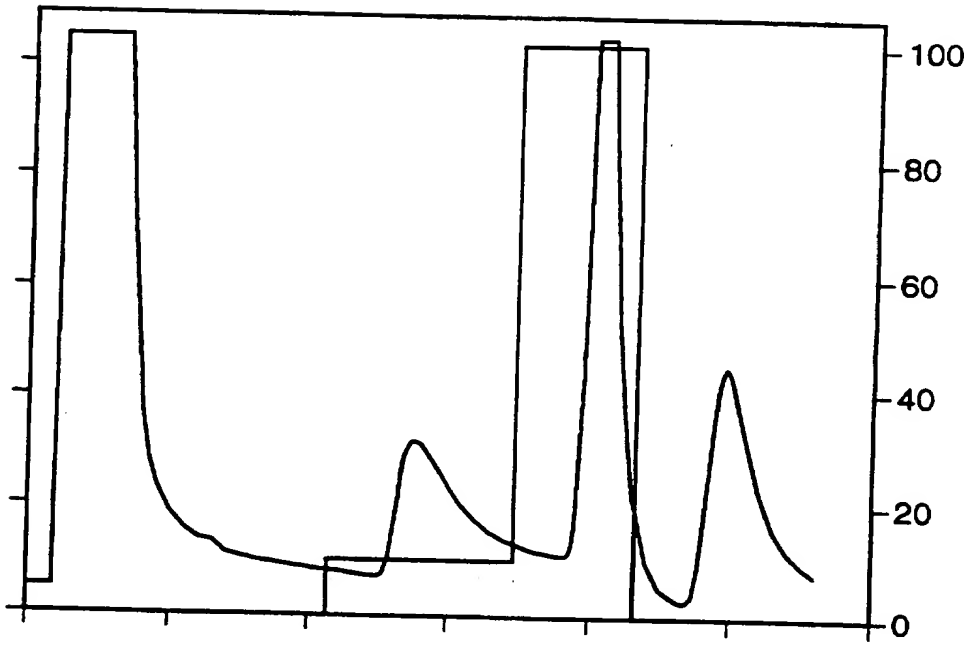
bSA

Figure 3B



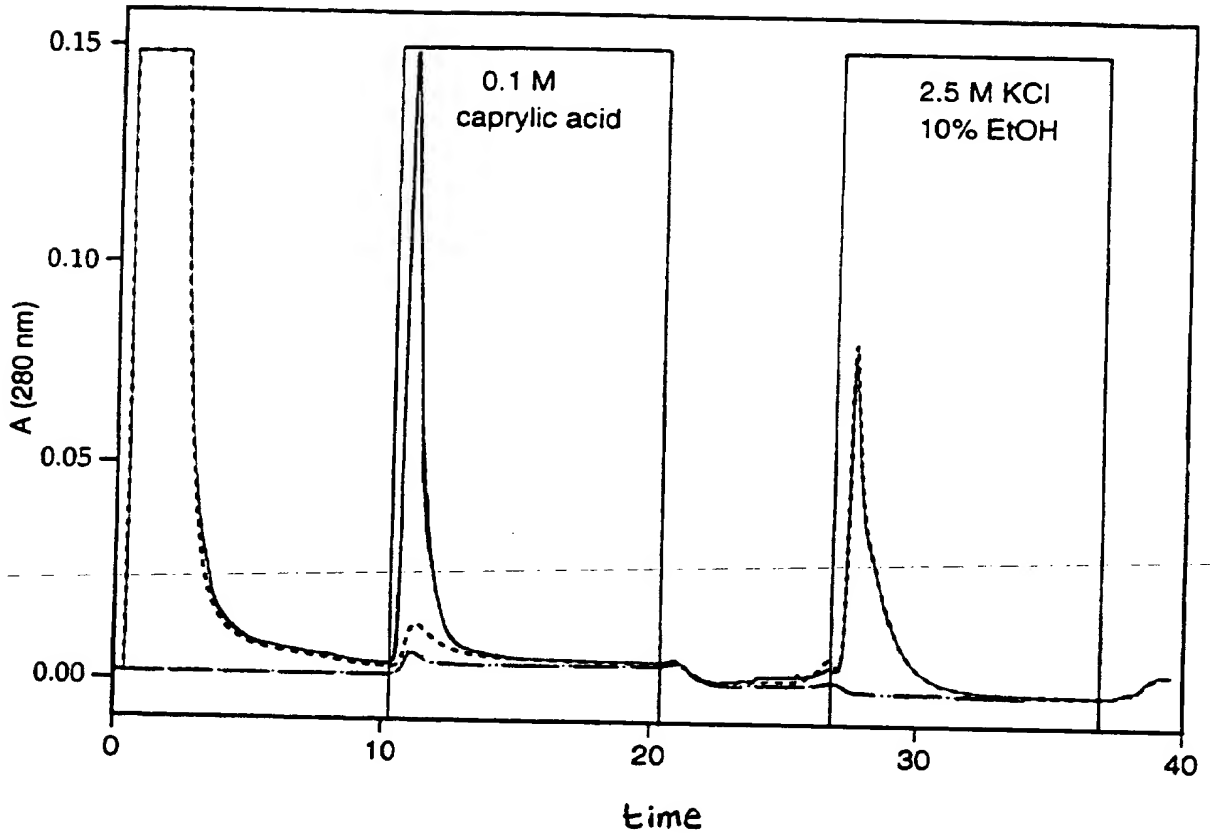
Whey

Figure 4A



Whey + hSA

Figure 4B



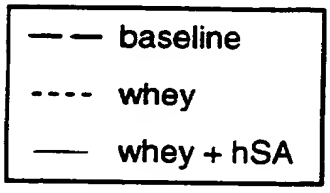


Figure 5A

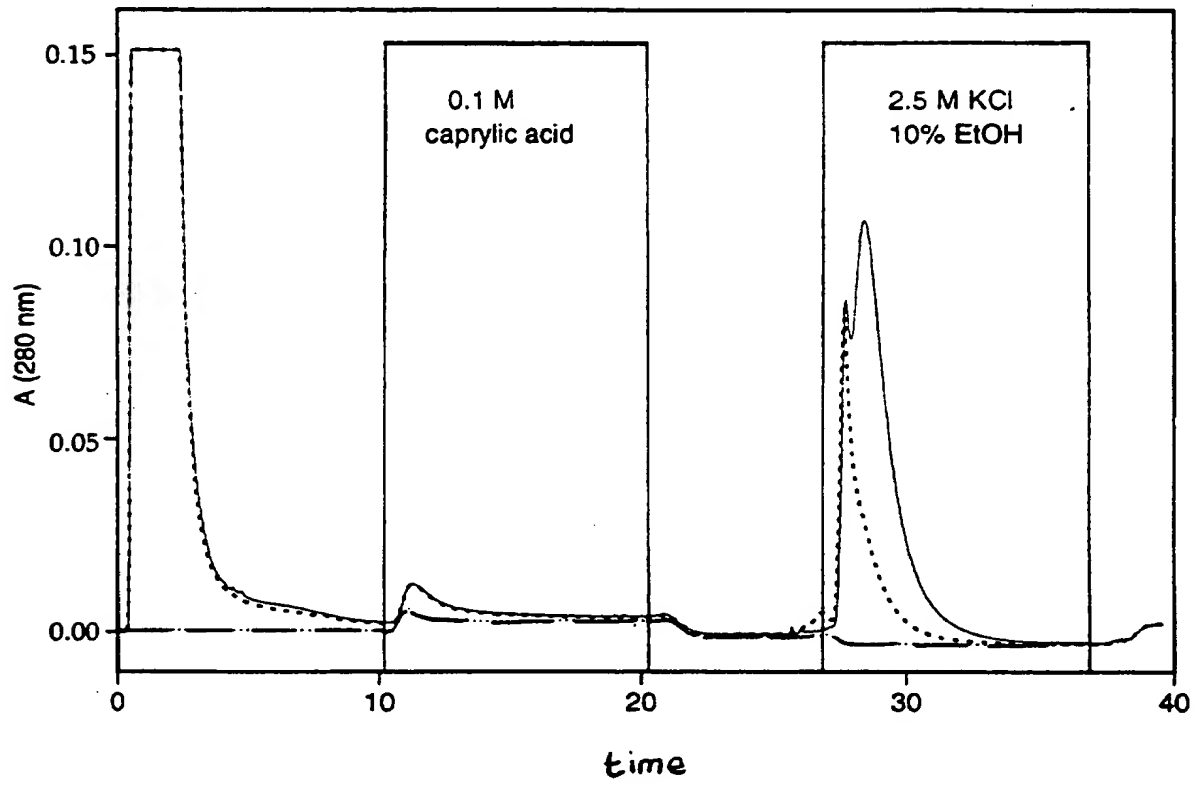


Figure 5B

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IB 95/00608

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/765

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 15887 (BAXTER DIAGNOSTICS INC.) 17 September 1992 see page 14 - page 16 ---	1-33
A	TRANSFUSION, vol. 27, no. 4, 1987 pages 302-308, KING M.J. 'Haemagglutination enhancement by bovine serum albumin is affected by octanoate, Reactive Blue 2 (Cibacron Blue), and polymer' see the whole document ---	1-33
A	WO,A,91 08216 (GENPHARM INTERNATIONAL) 13 June 1991 cited in the application see page 68 - page 70 --- -/--	1-33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 October 1995	29.11.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE BIOCHEMICAL JOURNAL, vol. 157, no. 2, August 1976 LONDON GB, pages 301-306, TRAVIS J. ET AL. 'Isolation of Albumin from Whole Plasma and Fractionation of Albumin-Depleted Palsma' cited in the application see the whole document	1-33

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter al Application No
PCT/IB 95/00608

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9215887	17-09-92	AU-B- 1239495	01-06-95
		AU-B- 1239695	22-06-95
		AU-B- 1239795	13-07-95
		AU-B- 648159	14-04-94
		AU-A- 1458392	06-10-92
		AU-B- 5499994	21-04-94
		EP-A- 0546127	16-06-93
		US-A- 5427949	27-06-95
		US-A- 5308459	03-05-94
WO-A-9108216	13-06-91	AU-B- 656720	16-02-95
		AU-B- 6960891	26-06-91
		CA-A- 2075206	02-06-91
		CN-A- 1053446	31-07-91
		EP-A- 0502976	16-09-92
		OA-A- 9669	15-05-93

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